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Deterministic or Stochastic Choices in Retinal Neuron Specification

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There are two views on vertebrate retinogenesis: a deterministic model dependent on fixed lineages and a stochastic model in which choices of division modes and cell fates cannot be predicted. In this issue of *Neuron*, He et al. (2012) address this question in zebrafish using live imaging and mathematical modeling.

The central nervous system (CNS) is composed of a highly diverse set of specialized neurons and glia that are derived from a much smaller population of progenitor stem cells. It is critical for the proper functioning of the nervous system that all types of neural cells be produced in the right numbers and proportions. Thus, we must understand how each progenitor cell generates progenies of different cell types and how the sum of all lineages reflects the repertoire of neurons found in a developed brain. Are progenitor cells multipotent? Are they already programmed to produce a fixed series of neural cell types or do they respond to extrinsic clues? How is the pattern of cell division of progenitors determined?

The development of the *Drosophila* CNS provides a great example of how an intrinsically programmed multipotent progenitor cell (neuroblast) generates specific neurons with a highly deterministic lineage (Figure 1A): each neuroblast divides asymmetrically multiple times to generate a self-renewing neuroblast and a series of ganglion mother cells (GMCs). In most cases, each GMC only divides once to generate two neurons or glia. As a neuroblast cycles through these divisions, it changes its competence to generate neural types. For example, as neuroblasts in the *Drosophila* ventral nerve cord divide, they sequentially express a temporal cascade of five transcription factors: Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2 (Pdm), Castor (Cas), and Grainyhead (Grh) (Brody and Odenwald, 2000; Isshiki et al., 2001). Those transcription factors are both required and sufficient for the neuroblast to generate a specific lineage of different neuron types in a defined order that can be recapitulated in vitro (Isshiki et al., 2001; Brody and Odenwald, 2000).

The vertebrate retina is a relatively well-characterized model to study similar questions. It is easily accessible for experimental manipulations during development. The retina contains only seven major cell types: retinal ganglion cells (RGCs), horizontal cells (HCs), bipolar cells (BCs), amacrine cells (ACs), Müller cells, cone photoreceptors (cone PRs), and rod photoreceptors (rod PRs). The seven cell types are born in a chronological order with significant time frame overlaps during retinogenesis (Livesey and Cepko, 2001). Pioneering analysis of RPC clone size and cell-type composition in murine retina showed that retina progenitor cells (RPCs) are multipotent and can give rise to multiple cell types with great variability in clone size and cell composition (Turner et al., 1990). These results led to the proposal of the "competence model" that suggests that RPCs undergo an irreversible series of states similar to the *Drosophila* neuroblasts. At each state, RPCs have different competence to produce one or a few cell types. The progression from one state to the next was proposed to be controlled intrinsically by sequentially expressed transcription factors. Those transcription factors would make RPCs capable of responding to extrinsic environmental signals and generate desired cell fates (Livesey and Cepko, 2001). The time frame overlaps for the production of various cell types during retinogenesis could be due to asynchrony among RPCs. Consistent with this model, Ikaros, a homolog of the *Drosophila* early temporal transcription factor Hunchback, is necessary and sufficient for the early temporal competence of mouse RPCs. Ikaros mutants show a reduction of early-born neural types but normal later-born cell types (Elliott et al., 2008). A variety of other transcription factors are expressed in later-stage RPCs (Trimarchi et al., 2008), but no clear big picture has emerged as to how the various cell types are generated sequentially.

The competence model has to explain how fixed lineages can be reconciled with the great variability in size and cell-type compositions of clones generated in vertebrate retina. It is possible that a combination of intrinsic competence states and varying extrinsic signals determines cell type and proliferation (Turner et al., 1990). However, in vitro experiments raised doubt that extrinsic signals from outside of the lineage have such a critical role in retinogenesis since lineages of RPCs cultured at sparse clonal density, when analyzed as a population, show the same clone size and cell composition distribution as an in vivo retina of the comparable developmental stage (Cayouette et al., 2003; Gomes et al., 2011).

If no extrinsic signal is required, then can the great variations of size and cell type in individual RPC lineages be determined intrinsically? There are two possible models: parallel predetermined lineages or stochastic choices (Cayouette et al., 2003). In the first model, variation may be due to the existence of multiple types of RPCs, and thus multiple fixed lineages that differ between them but are each deterministic (Cayouette et al., 2003; Livesey and Cepko, 2001). Indeed, there is huge heterogeneity of the transcriptome of individual RPCs in the

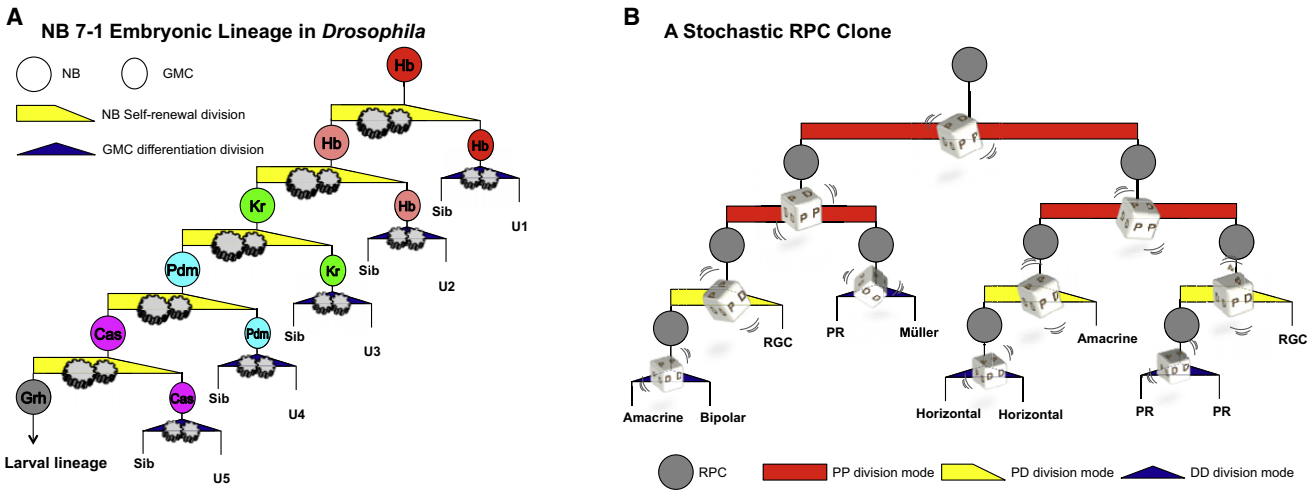

Figure 1. Comparison between a *Drosophila* Neuroblast Lineage and a Conceptual Zebrafish Retinal Progenitor Cell Lineage
(A) The *Drosophila* embryonic ventral nerve cord NB 7-1 lineage (after Pearson and Doe, 2004). As the NB undergoes several rounds of asymmetrical divisions, it sequentially expresses five transcription factors: Hb, Kr, Pdm, Cas, and Grh. The lineage of this specific NB is predetermined.
(B) In vertebrate retinogenesis, there is no predefined order of modes of cell division. In the zebrafish lineages analyzed by He et al. (2012), RPCs stochastically choose one of three modes of division (PP, PD, and DD). As retinogenesis progresses, RPCs shift from mostly PP divisions to PD and DD divisions. The neural cell-type decisions appear to also be largely stochastic.

population (Trimarchi et al., 2008). Selective expression of certain transcription factors can also restrict the spectrum of cell types in subsets of RPCs. For example, in zebrafish, Vsx2 initially expressed in all early RPCs is downregulated in subsets of RPCs to allow the expression of transcription factors that restrict lineage potentials, such as Vsx1, Ath5, or Foxn4. Among them, Ath5 restricts RPCs to generate RGCs, HCs, ACs, cone PRs, and rod PRs, while Foxn4 is expressed in RPCs that generate ACs and HCs, and Vsx1 is present in RPCs that generate BCs (Vitorino et al., 2009). However, it is still not clear how the expression of these earlier transcription factors is regulated. The parallel lineages model would also require that the expression pattern of these transcription factors not be random but instead be identical among RPC subsets between individual animals. It will therefore be necessary to characterize more subtypes of early RPCs to ensure that some share identical lineages.

In the stochastic model, a given RPC does not have a predefined pattern of mitosis or progeny fate specification. Its lineage is the result of random choices of cell fates made at each cell division by the progeny. It might be difficult to imagine that stochastic lineages from progenitor cells can generate homeostatic tissues with consistent size and cell-type composition. However, studies in other stem cell model systems suggest that this is possible. For example, quantitative analysis showed surprising stochasticity in the progeny of stem cells in self-renewing adult tissues such as the murine epidermis and intestinal epithelium (reviewed in Simons and Clevers, 2011). In these systems, the stem cells do not follow the classic asymmetrical self-renewing division mode. Instead, they usually divide symmetrically and the resultant progeny make their own stochastic choices to stay in the stem cell fate or to move toward a differentiated cell fate. Although this stochasticity results in great variation in the size, cell-type composition, and dynamics of individual stem cell clones, modeling showed that the various cell types can be produced in the correct proportion, while tissue homeostasis can be well maintained at the population level (Simons and Clevers, 2011).

Which model better fits the actual vertebrate retinogenesis scenario? Statistical analysis and mathematical modeling of data from in vitro cell culture and time-lapse microscopy had unveiled similar stochasticity in late rat RPCs (Gomes et al., 2011), which choose to divide with three possible outcomes with a specific proportion of each division mode at a given stage of development. These modes give rise to (1) two daughter progenitor cells (PP division), resulting in expansion of the progenitor population; (2) one progenitor daughter cell and one differentiating daughter cell (self-renewing PD division), which is a stem cell mode that produces neurons with a linear amplification; and (3) two terminally differentiated daughter cells (DD division), a mode that ends the lineage (Figure 1B). The variability in the cell-type birth order and the inability to identify a large number of identical lineages also showed that the system might rely on stochastic choices of cell fates. However, there were still important questions remaining. Is the stochastic model true in vivo and is it applicable to earlier-stage RPCs?

The paper by He et al. (2012) addresses these questions in zebrafish by tracing RPC lineages in vivo in the developing retina. Zebrafish are an excellent model organism for this purpose as their retinas are easily accessible for manipulation and allow live imaging even at early retinogenesis stages. Using photoconvertible fluorescent protein expression in clones induced by heat shock, He et al. (2012) monitored lineage progression, progeny fate decisions, and cell-cycle durations

for many individual RPCs through retinogenesis. This rendered possible mathematical analysis and precise modeling of a developing in vivo vertebrate CNS structure. These analyses showed that, as RPCs progress through multiple mitoses, they exhibit a reduction of their cell division rate and a shift from the preferred PP division mode to the PD and finally the DD division modes (Figure 1B). The observed clones, as a population, faithfully represent the proliferation dynamics of the whole retina. However, individual clones show great variations in the size and division mode dynamics. Based upon these observations, He et al. (2012) built a simple mathematical model in which cells make probabilistic mode choices at each division (Figure 1B). This stochastic model can precisely predict the clonal size distribution as well as the division mode distribution observed at different time points in their experiments.

During retinogenesis, different cell types are born in a sequential order with significant overlap. When analyzed at the population level, the live-imaging data from the in vivo zebrafish RPC clones are consistent with the known birth order. However, when individual clones are examined, there is no strict birth order of different cell types (Figure 1B). Innovative barcode analysis of lineage similarity also supports the stochastic model. Further analysis revealed that the generation of certain cell types seems to correlate with specific types of division modes. For example, most RGCs arise from the D cell of PD divisions. ACs arise from both PD and DD divisions, while BCs, HCs, rod PRs, and cone PRs are mostly associated with DD divisions. Therefore, the birth probabilities of different cell types vary as RPCs progress through cell cycles and change their stochastic preference of division modes, which suggests that there could be connections between certain cell fate choice and division modes.

In support of this "connection proposition," He et al. (2012) discovered that Ath5 acts as a molecular link between the mode of division and cell-type specification. RGCs are born earlier than other retinal cell types. Ath5, a gene previously shown to be required for the specification of RGCs, is also crucial for the PD division mode. Ath5 mutations or knockdown cause a delay of retinal differentiation and an increase in retinal size and RPC clone size, which corresponds to what is predicted by a change of the PD divisions that generate RGCs to the amplifying PP mode of division. This finding connects retinogenesis order with the stochastic model and explains why RGC differentiation is always earlier than that of other neural types.

However, the paper by He et al. (2012) also raises intriguing new questions. When comparing in vitro data from rat to in vivo data from zebrafish, one striking difference is that cultured rat RPCs show more or less stable ratio of division modes across cell cycles, while zebrafish RPCs change the probabilities of modes of cell division as they progress through cell cycles. One possible explanation is that isolated rat RPCs used in the previous study were relatively late in retinogenesis and were already dominated by PD and DD division modes. However, the number of cell cycles of some in vitro rat RPC lineage trees is similar to that of zebrafish RPCs, suggesting that they might not be that late. Thus, it will be interesting for future research to compare side-by-side stochastic retinogenesis models between these two systems in a more stringent way and to look for both conserved features and dissimilarities.

Although the great variation in individual RPC lineages seems to contradict a deterministic programming model and instead favors the stochastic model, this does not mean that the regulation of RPCs and their progeny is completely without any deterministic elements in fate choice. For example, in the two progeny from DD divisions of zebrafish RPCs, the same cell-type combinations of BCs, HCs, and PRs are produced at much higher frequencies than predicted by pure unbiased stochastic choices (He et al., 2012). Similarly, in rat RPCs in vitro, certain cell-type choices in two successive RPC divisions might not be completely independent (Gomes et al., 2011). Furthermore, a dedicated subpopulation of zebrafish RPCs has been shown to divide symmetrically to generate exclusively BCs (Godinho et al., 2007). These examples illustrate how much deterministic inputs might bias the stochastic choices. Such inputs are probably from those genes differentially expressed in RPCs that regulate progeny cell fates. For example, as mentioned above, the expression of Vsx1, Vsx2, Foxn4, and Ath5 is important for restricting progeny fates of RPC subpopulations (Vitorino et al., 2009). Furthermore, mouse NeuroD6, a member of the atonal-like family of bHLH transcription factors, is critical for AC fate choice as forced NeuroD6 expression leads to significant increase in ACs (Cherry et al., 2011). In mice, Olig2$^+$ RPCs, which appear later in RPC lineages, usually divide in DD (terminal) mode but the fate of the progenies varies over time: embryonic Olig2$^+$ RPCs are biased toward generating cone PRs and HCs, while postnatal Olig2$^+$ RPC progenies are enriched for rod PRs and ACs (Hafler et al., 2012). The high heterogeneity of RPC transcriptomes (Trimarchi et al., 2008) suggests that there are more examples of such genes waiting to be characterized.

Future research will have to understand the mechanisms that regulate the expression of these transcription factors and whether their expression is strictly controlled by temporal and/or by spatial patterning. This would suggest a general deterministic control. If the regulations of these cell fate genes were to show typical stochastic features, this would provide further support for the stochastic model. Probably, a combination of deterministic and stochastic features will govern patterning of this complex structure.

In fact, although the choices described above only involve seven major retinal cell types, the diversity of neuron subtypes within these major types is enormous in the vertebrate retina. For instance, there are 8–10 subtypes of BCs, at least 28 subtypes of ACs, about 12 subtypes of RGCs, and 3 subtypes of HCs. Each subtype has a distinctive morphology and arborization pattern (reviewed in Masland and Raviola, 2000) and might depend on specific patterning mechanisms. For instance, in the chick retina, clones induced late in development contain only homotypic pairs of horizontal cell type 1, or of type 3, but not of type 2 (Rompani and Cepko, 2008). Therefore, it will be critical in the future to take into account the subtypes and to increase the "resolving power" of the modeling of

cell fate choices. More subtype-specific molecular markers will need to be identified, progresses in automatic image acquisition and in techniques to reliably identify cellular subtypes in clones and cell cultures will be required, and sophisticated mathematic modeling of cell fate choices based on a biased stochastic division will also be required. These advances will probably lead to an integral model combining both stochastic and deterministic inputs.

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The Clathrin Adaptor Complex Responsible for Somatodendritic Protein Sorting

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Neuronal proteins contain "address labels" that govern their localization. In this issue of *Neuron*, Farías et al. (2012) identify the machinery that recognizes one class of dendritic localization signals and establish its role in the polarization of dendritic proteins, including several postsynaptic receptors.

Nearly every aspect of neuronal function depends on the accurate trafficking of membrane proteins to specific sites within the axon or dendrites. While the complexity of protein targeting in neurons is extraordinary and neuronal dimensions are extreme, the basics of neuronal protein sorting are shared with many other polarized cells, such as epithelial cells. Many advances in understanding neuronal protein targeting have come from exploiting parallels between the two systems, a strategy first put forward by Dotti and Simons (1990).

In epithelia, the cytoplasmic domains of basolateral proteins contain short, linear motifs, including YxxΦ (where Φ is a bulky hydrophobic residue), and dileucine motifs, which direct their sorting. Near the end of the last millennium, parallel studies of neuronal proteins led to the first identification of dendritic sorting signals (Jareb and Banker, 1998; West et al., 1997). Based on work from many groups that have studied the localization of proteins in cultured neurons (reviewed by Horton and Ehlers, 2003; Lasiecka et al., 2009), as well as in transgenic animals (Mitsui et al., 2005), a clear picture has emerged: dendritic proteins contain sorting signals located within their cytoplasmic domains. Some of these signals resemble the YxxΦ motifs identified in basolateral proteins. Interestingly, dihydrophobic motifs that mediate basolateral sorting are not always sufficient for dendritic sorting (Silverman et al., 2005). What machinery recognizes these targeting signals to ensure that dendritic proteins are sorted into a distinct vesicle population? Many sorting events depend on clathrin adaptor proteins, which bind to and recruit cargo proteins to sites of vesicle budding. With the discovery that a novel form of the clathrin coat adaptor AP-1 (containing a distinct μ1B subunit) plays a critical role in basolateral sorting (Fölsch et al., 1999), the elucidation of the machinery for dendritic sorting seemed to be only a matter of time. This expectation turned out to be far too optimistic. It was soon established that AP-1B is not expressed in neurons, and, as the new decade dragged on, the machinery responsible for recognizing dendritic sorting signals remained as mysterious as ever. In this issue, Farías